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Determination of theobromine, theophylline and caffeine in cocoa samples by a high-performance liquid chromatographic method with on-line sample cleanup in a switching-column system

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Abstract

A simple reversed-phase high-performance liquid chromatographic method was developed for the determination of theobromine, theophylline and caffeine in cocoa samples. In the sample cleanup step, the procedure involves an on-line solid-phase extraction of analytes from cocoa samples into a home-made dry-packed pre-column with ODS-C_{18} using a column-switching system. The separation was performed on a C_{18} Nova-Pak column (150 mm × 3.9 mm, 4 μm) using a mobile phase consisting of a solution of 20% of methanol in water under isocratic conditions, at a flow-rate of 1.4 ml/min. The validation method revealed quantitative recoveries (>95.0%) with a coefficients of variation <3.2% and it also provided a good precision for data validation. The overlap of sample cleanup, analysis and recondition of the precolumn increases the sample throughput to 8 samples/h. Furthermore, the proposed method was successfully applied to analysis of cocoa samples "Trinitario", "Forastero" and "Criollo" grown in different seasons of the year and fermented for 3 and 7 days, respectively. The results showed a slight reduction in the theobromine and caffeine content according to the fermentation times. In the same way, the theobromine/caffeine ratio was assessed, with the purpose of establishing a correlation with the genotype of the studied samples.

Keywords: Cocoa; Theobromine, Caffeine; Theophylline; HPLC; Solid-phase extraction; Column-switching

1. Introduction

The quality of the cocoa beans depends on many factors such as the genotype, the agronomic management, the soil factors, the climatic conditions, and most importantly the post-harvest technology. In this way, the quality of cocoa beans' taste and aroma will depend on the skills and good care taken by the technicians in charge. It is important to mention that Venezuelan and international communities have been implementing better quality measures to improve the cocoa competitively. The current measurements and quality criteria do not objectively reflect the fine taste attributes; they increase the problems of manufacturers and dealers face in classifying and standardizing their products. Because of this, it is necessary to evaluate physical, chemical and organoleptic parameters, which allow us to determine the cocoa's quality regarding the genotype and the environment.

In order to improve our understanding of cocoa, we have initiated a study about Venezuelan cocoa beans. It is therefore important to analyze the methylxanthines (theobromine, theophylline, and caffeine), because their levels depend on the genotype and affect the flavour of the cocoa beans. Different analytical techniques have been

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developed for the simultaneous determination of these three methylxanthines. Liquid chromatography is, however, the most frequently method used nowadays. Most of the previously employed procedures were based on reversed-phase chromatographic separation with ultraviolet absorbance detection (Bispo et al., 2002; Hurst, Snyder, & Martin, 1985; Parra, Limon, Ferre, Guix, & Jane, 1991; Pura Naik, 2001; Terada & Sakabe, 1984; Thomas, Yen, Schantz, Porter, & Sharpless, 2004; Vergnes & Alary, 1986; Zambonin, Aresta, & Palmisano, 2004), mass spectrometric detection (Hieda, Kashimura, Hara, & Kageura, 1995) and amperometric detection (Meyer, Ngiruwonsanga, & Henze, 1996). The separation of these compounds could also be accomplished by ion-pair, or ion-interaction, or ion chromatography coupled with UV detection (Blanchard et al., 1990; Gennaro and Abrigo, 1992; Gennaro et al., 1992; Lauff, 1987; Qin-Chuan Chen & Jing Wang, 2001; Qin-Chuan Chen, Shi-fen Mou, Xiao-ping Hou, & Zhe-ming Ni, 1998). In most cases, however, these methods involve tedious and laborious pre-treatment steps before the chromatographic determination. The extraction of these methylxanthines has been performed using liquid extraction solvents such as dimethyl chloride, chloroform and water (Caudle, Gu, & Bell, 2001; Hulbert, Biswal, Walker, Meher, & Collins, 1998). However, chemical solvents require several hours for a complete extraction to take place. Water, although an excellent solvent of methylxanthines, is highly non-selective and its use may result in the removal of other valuable components from the extracted product, which gradually leads to deterioration of the analytical column (Saldana, Zetzl, Mohamed, & Brunner, 2002). In order to resolve this problem, Pura Naik (2001) proposed the use of the Seppak C_{18} cartridge for the purification of cocoa extract before injection onto a HPLC C_{18} reverse-phase column. In this way, the interfering cocoa pigments are effectively removed, therefore increasing the column life. Nevertheless, this cleanup procedure can be time-consuming, because it has to be carried out manually and also need a step of evaporation of the solvent ($CHCl_3$) used to elute the analytes from the cartridge. In the last decades, on-line column-switching devices, combined with pre-column packed with different kinds of materials have been shown to provide a powerful and reliable solution to the on-line sample treatment of complex matrixes. It is therefore possible to inject the sample directly into the chromatographic system, avoiding the problems associated with off-line sample pretreatments, such as time-consuming procedures, errors and the risk of low recoveries.

The aim of the present investigation was to develop a method for the determination of theobromine, theophylline and caffeine in cocoa beans samples using high-resolution reversed-phase liquid chromatography with UV diode array detection, and with on line sample cleanup in a column-switching system. The proposed procedure offers several advantages which include simplicity and short analysis time, low cost, and high-sample throughput.

2. Materials and methods

2.1. Reagents and standards

All solvents and reagents used were HPLC or analytical reagent grade, unless indicated otherwise. Methanol and ethanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was purified (18 $M\Omega\,cm^{-1}$quality) by a Milli-Q system (Millipore, Bedford, MA, USA). Theobromine, theophylline and caffeine were supplied by Sigma (St. Louis, MO, USA).

Individual stock standard solutions of theobromine (400 μg/ml), theophylline (1000 μg/ml) and caffeine (1000 μg/ml) were prepared by dissolving appropriate amounts of these compounds in water and subsequently stored at 4 °C. Working solutions were prepared every week by diluting of concentrated stock standard solutions in water.

2.2. Instrumentation and chromatographic conditions

The chromatographic analysis was performed on a liquid chromatographic system equipped with a Waters Alliance 2690 HPLC module (Milford, MA, USA) connected to a Waters 996 photodiode array (PDA) detector and with an additional pump, Waters model 515 used to deliver mobile phase for the analysis. Data were collected, stored and analyzed using the MILLENIUM software version 3.2 from Waters (Milford, MA, USA). Injections were made with the autosampler of the Alliance module. Column-switching was performed by a LabPro column-switching six-port valve (Waters) controlled by the workstation. On-line extraction was carried out using a homemade dry-packed precolumn (50 mm × 4.6 mm i.d.) with Alltech ODS-C18 (15–40 μm). Chromatographic separation was achieved on a NOVAPAK C_{18} column (150 × 3.9 mm I.D., 4 μm particle size, from Waters) maintained at room temperature (22 °C). A diagram of the different column-switching positions in the HPLC system is shown in Fig. 1.

A solution of 20% methanol in water (v/v) at a flow-rate 1.4 ml/min was used as the mobile phase to transfer and separate the analytes. The elution conditions were isocratic. Before used, the mobile phases were vacuum-filtered through a 0.45 μm nylon filter and degassed. The chromatograms were monitored by UV detection at a wavelength of 274 nm.

2.3. Cocoa beans preparation

In order to apply the proposed method, ''Criollos'', ''Trinitarios'' and ''Forasteros'' cocoa beans subject to several stages of fermentation (1–7 days) were analyzed.

Cocoa pods from variety ICS-1 (Trinitario) and IMC-67 (Forastero) were obtained from genetically identified trees grown on the Cocoa Genebank of Ocumare de la Costa (INIA-Aragua, Venezuela). ''Guasare'', ''Criollo Merideño Zea'' and ''Criollo Merideño San Juan'' (SJN) were

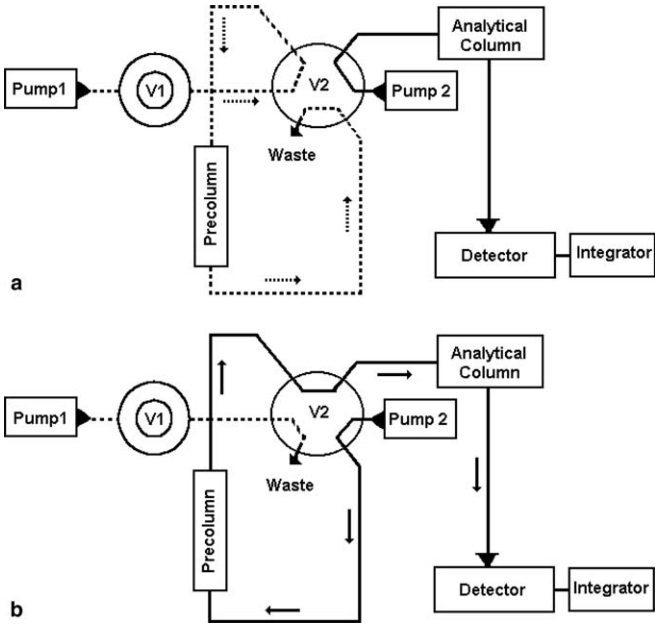

Fig. 1. Flow diagram for switching-column system. V_1, injection valve; V_2, switching valve; (a) V_2 in loading position; (b) V_2 in injecting position. Flow direction is indicated by arrows. Description in text.

furnished from the Cocoa Genebank of San Juan de Lagunillas (INIA – Mérida, Venezuela), and Porcelana were obtained on the Cocoa Genebank of the Local Chama Station (CORPOZULIA, Venezuela). Pods were collected in intervals of two crop cycles yearly. The harvested pods were stored under refrigeration at −20 °C.

On the other hand, during the optimization of the developed procedure, a ''Forastero'' cocoa sample from Ghana was used as reference material.

After fermentation and drying, the shells of the cocoa beans were removed manually and 100 g of them were weighed out. The beans were then ground in a rotating blade mill and the powder obtained was finally passed through a sieve of 45 mesh. Six grams of cocoa powder were weighed out, placed inside a thimble, covered with fat free cotton and the thimble was introduced into a clean Soxhlet extractor. After this, 50 ml of petroleum ether were transferred into a weighed and dried distillation flask, it was connected to the Soxhlet extractor and afterwards, 100 ml of the same solvent were additionally added until it started to siphon. Finally, the condenser was attached to the extractor and the complete system was placed on the heating mantle and fat was extracted from the cocoa powder for at least 4 h. A complete blank extraction (without cocoa powder) has been regularly performed in order to ensure that the blank residue was less than 2.5 mg (ICCO, 1996).

2.4. Extraction procedure

The powdered and fat-reduced cocoa samples were extracted by means of the following procedure. 0.0100 g of powder was extracted with approximately 10.00 ml of hot water (80 °C) in a heated circulating bath during 20 min. The solution was then cooled down to room temperature, it was centrifuged at 3000 rpm, and the supernatant was filtered through a Millipore filter of 45 μm of pore diameter. Subsequently, 20 μl of this solution were injected into the chromatographic system for the cleanup procedure and determination.

2.5. Sample analysis

After the extraction procedure the analytes were present in a complex mixture, and a sample cleanup was needed in order to preserve the analytical column.

The cleanup and separation of theobromine, theophylline and caffeine in cocoa samples were performed by a column-switching procedure, which can be subdivided into three different steps. *Sample loading step*: 20 μl of cocoa sample extract were loaded into the pre-column with the cleanup mobile phase composed of an aqueous solution of 1% (v/v) of methanol at a flow-rate of 0.8 ml/min. During this time, the purines were retained while others endogenous constituents were passed through the pre-column to waste (Fig. 1(a)). *Analytes transfer step*: Then, 3.0 min later, the analytes were back-flushed from the precolumn and directed to the analytical column with the mobile phase for the analysis: 20% (v/v) methanol in water at a flow rate of 1.4 ml/min (Fig. 1(b)). Continuously, after one min of transfer-time, the switching valve returned to the load position and the cleanup mobile phase flowed through the precolumn in order to equilibrate it prior to the next injection. *Separation step*: Simultaneously, the analytes continued the separation processes in the analytical column and they were UV detected (274 nm) at the exit. The total run time was 10 min.

3. Results and discussion

3.1. On-line sample clean-up and chromatographic separation

At present, HPLC is the best tool to determine purine alkaloids in cocoa extracts. From the viewpoint of molecular structures (shown in Fig. 2), the three alkaloids can be separated by reversed-phase liquid chromatography on a C_{18} reversed-phase column using a UV detector. Although several detection modes have been recently proposed, UV detection is still the first choice in the liquid chromatography

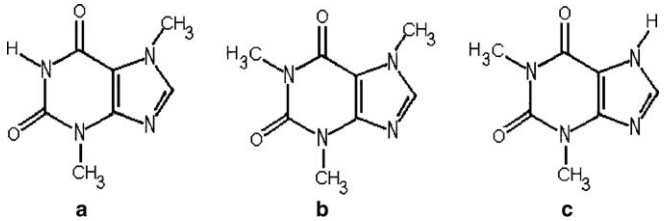

Fig. 2. Chemical structures of the methylxanthines determined in this study. (a) theobromine; (b) caffeine; (c) theophylline.

analysis of these analytes due to its simplicity and reliability. In this work, the maximum absorption wavelengths is 274 nm for the three compounds, which facilitate the simultaneous determination of all the analytes. On the other hand, the background absorbancies of eluents were very low at 274 nm, so, this measurement was chosen for further studies.

The solubility of theobromine, theophylline and caffeine is very much related to temperature (Macrane, 1985), which favors the solubility of many other compounds such as the pigments. In this sense, these compounds were present in the filtrate during the extraction procedure (Section 2.4) interfering in the HPLC analysis and being able to reduce the lifespan of the analytical column if it were not removed before the injection onto the HPLC system (Pura Naik, 1997). In order to solve this problem, an on-line solid phase extraction procedure was performed using a column-switching system. This system is characterized by four steps: sample cleanup, analyte transfer, separation, and reconditioning. In this way, the sample cleanup step was carried out in a home-made precolumn (50 × 2 mm) dry-packed with Alltech ODS-C_{18} phase, 15–40 μm particle diameter. The precolumn was first preconditioned by pumping 10 mL of methanol. Aiming to obtain more selectivity, so as to obtain quantitative recoveries of analytes during the extraction step, the nature and composition of the extraction mobile phase were optimized. The best result was obtained with an extraction mobile phase composed of 1% methanol in water (v/v) at a flow-rate of 0.8 ml/min during 3 min. At this time, most of the sample matrix had been eluted from the precolumn; however, theobromine, theophylline and caffeine still remained. It is important to point out that the addition of limited amount of an organic modifier, such as methanol, to the washing liquid is very useful in order to enhance the extraction selectivity and to obtain high recoveries from the analytes. Finally, the efficiency of the selected washing liquid to wash out the sample matrix was tested by injecting 20 μl of the treated Ghana cocoa sample into the precolumn directly connected to the UV-DA detector and monitored at 274 nm. The elimination of the matrix could be considered as complete when the detector signal reached the baseline. Under these conditions, a washing time of 3 min was enough for the sample cleanup and gave rise to no loss of analytes, since its breakthrough time for the first eluted analyte was more than 4 min (Fig. 3).

In the transfer step, the retained analytes were transferred from the precolumn to the analytical column by means of analytical mobile phase. Peak compression of the analytes eluting in backflush mode from the precolumn could be achieved by ensuring that the percentage of the organic modifier used to transfer and separate was higher than the one in the extraction mobile phase. The composition of the analytical mobile phase was also chosen in a way that enough selectivity could be obtained in the final separation of theobromine, theophylline and caffeine in the analytical column. Different mixtures of organic solvents in water were studied as analytical mobile phase. A mixture of 20% of methanol in water (v/v) under isocratic conditions at a flow-rate of 1.4 ml/min reported an excellent resolution between the analytes and a short analysis time. However, it was found that the purines adsorbed in the extraction precolumn were completely eluted out in 1.0 min by the analytical mobile phase. After this time, the valve was switched back to the loading position (Fig. 1(a)) in order to recondition the extraction pre-column with the extraction mobile phase for the next sample injection. The optimized conditions for the procedure and the times of the column-switching valve for on-line sample clean-up and chromatographic separation are indicated in Tables 1 and 2. Under these conditions, theobromine, theophylline and caffeine retention times were 5.22, 6.32 and 8.67 min, respectively. Fig. 4 shows the representative chromatograms obtained when the optimized proposed method was used for the analysis of a standard aqueous solution of analytes and of a cocoa sample spiked with the analytes. Comparing these figures, it can be concluded that both chromatograms are free from endogenous components and the separation of theobromine, theophylline and caffeine were completed with a reasonable assay time.

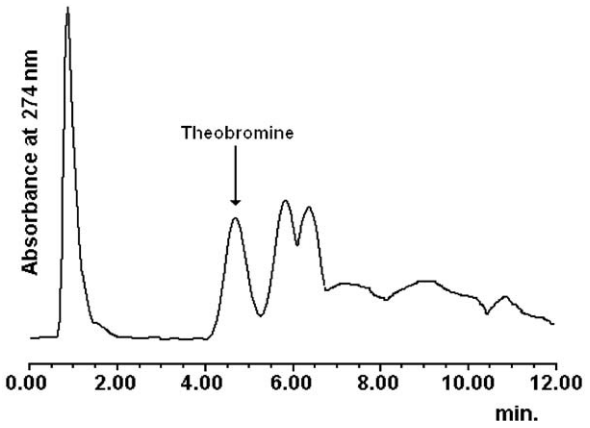

Fig. 3. Precolumn elution profiles for 20 μl of a cocoa sample spiked with theobromine, theophylline and caffeine. Experimental conditions in Tables 1 and 2.

Table 1
Optimal instrumental conditions for the determination of theobromine, theophylline and caffeine in cocoa samples using on-line sample cleanup

Sample injection volume	20 μl
Extraction precolumn	Homemade ODS-C_{18} (particle size: 15–40 μm) 50 × 2 mm internal diameter
Extraction mobile phase	1% methanol in water (v/v)
Flow-rate	0.8 ml/min
Analytical column	NOVAPACK C_{18}, 150 × 3.9 mm; internal diameter; particle size: 4 μm
Mobile-phase for analysis	20% methanol in water (v/v)
Flow-rate	1.4 ml/min
Temperature	Room temperature (22 °C)
Detection	UV-DAD at 274 nm

Table 2
Time events of the column-switching valve for on-line sample clean-up and chromatographic separation

Step	Process	Switching time (min)	Switching valve coupling
1	Sample clean-up	0–3	Precolumn-waste
2	Elution and transfer	3–4	Precolumn-analytical column-UV DAD detector
3	Equilibration of the precolumn and analytical column separation	4–10	Precolumn-waste
4	Next injection	7	

3.2. Validation of the method

The accuracy of the proposed procedure was evaluated by means of recovery experiments carried out from spiked samples at different analyte concentrations levels and also a blank constituted by the unspiked sample was analyzed. The concentration studied were prepared in five replicates from 2.00 to 20.00, from 0.50 to 10.00 and from 1.00 to 10.00 μg/ml of theobromine, theophylline and caffeine respectively. In all cases, the recovery percentage values ranged between 95.0% and 104.0% (Table 3) with a relative standard deviation <3.20% ($n = 5$). These values demonstrate a good extraction efficiency of the homemade ODS-C_{18} precolumn in the cleanup step, just like all the studied analytes were recovered quantitatively from the cocoa matrix.

The intra-assay (intra-day) and inter-assay (inter-day) variability of the method was assessed by analyzing the aqueous standards of theobromine, theophylline and caffeine and cocoa samples spiked with known amounts of analytes. The precision was evaluated through intra-day and inter-day percent relative standard deviation percentage (RSD) for five different concentration levels ranged from 2.0 to 20.0, 0.5 to 10.0 and 1.0 to 10.0 μg/ml for theobromine, theophylline and caffeine, respectively. The data is listed in Table 4. These results (RSD < 2.98% for all cases) confirm that good precision can be attained with the on-line cocoa sample cleanup described above.

Table 3
Recoveries of theobromine, theophylline and caffeine from cocoa samples

Compound	Concentration added (μg/ml)	Recovery (%)	CV ($n = 5$) (%)
Theobromine	2.00	102.3	3.20
	4.00	102.1	3.12
	8.00	104.0	2.80
	12.00	102.9	2.34
	20.00	102.2	1.90
Theophylline	0.50	95.0	2.74
	1.00	96.7	1.98
	2.00	97.5	2.54
	5.00	97.7	2.38
	10.00	99.8	2.85
Caffeine	1.00	95.1	2.99
	2.00	101.0	1.89
	4.00	98.9	2.90
	5.00	97.0	2.71
	10.00	97.5	3.10

CV, coefficient of variation ($n = 5$).

The linearity of the assay was performed with an eight point calibration curve prepared by diluting stock analytes solutions in water, and with cocoa powder spiked with theobromine, theophylline and caffeine to yield concentrations over the range from 2.0 to 20.0, from 0.5 to 10.0 and

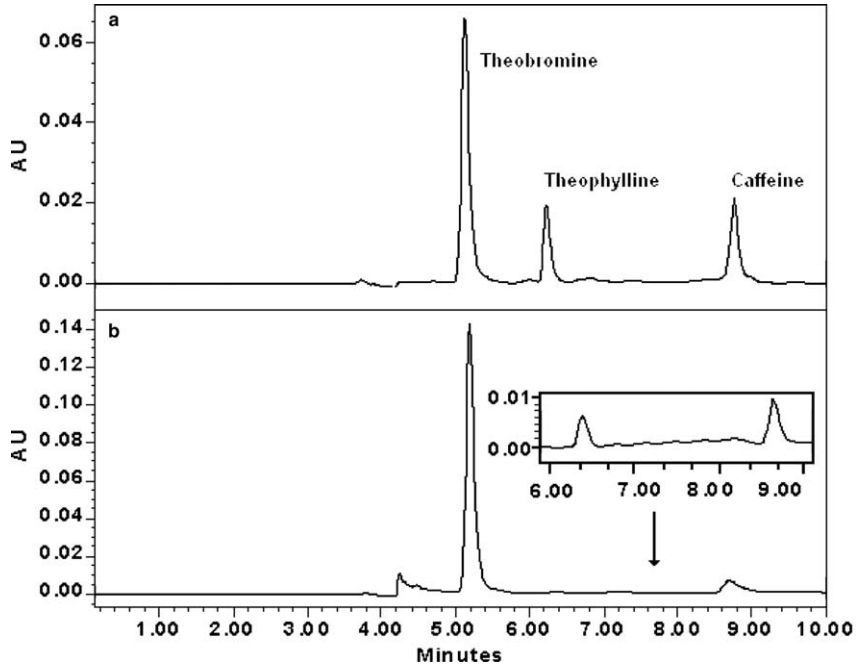

Fig. 4. Typical chromatogram obtained for: (a) aqueous standard solution of theobromine, theophylline and caffeine at 8.0, 2.0 and 2.0 μg/ml, respectively; (b) cocoa sample. Optimized conditions Tables 1 and 2.

Table 4
Results of precision studies for theobromine, theophylline and caffeine

Compound	Matrix	Within-day ($n = 5$)		Between-day ($n = 5$)	
		Concentration (mean ± SD) (μg/ml)	CV%[a]	Concentration (mean ± SD) (μg/ml)	CV%
Theobromine	Aqueous standard	2.000 ± 0.011	1.50	2.000 ± 0.011	1.73
		8.000 ± 0.003	1.20	8.000 ± 0.003	1.31
		20.000 ± 0.003	1.90	20.000 ± 0.003	1.95
	Cocoa matrix	2.000 ± 0.011	1.10	2.000 ± 0.011	1.82
		8.000 ± 0.003	1.30	8.000 ± 0.003	2.00
		20.000 ± 0.003	2.60	20.000 ± 0.003	2.80
Theophylline	Aqueous standard	0.500 ± 0.009	1.80	0.500 ± 0.009	1.80
		2.000 ± 0.013	1.30	2.000 ± 0.013	1.30
		10.000 ± 0.036	1.80	10.000 ± 0.036	1.80
	Cocoa matrix	0.500 ± 0.009	1.10	0.500 ± 0.009	1.80
		2.000 ± 0.013	2.30	2.000 ± 0.013	2.60
		10.000 ± 0.036	2.60	10.000 ± 0.036	2.75
Caffeine	Aqueous standard	1.000 ± 0.006	1.20	1.000 ± 0.006	1.59
		4.000 ± 0.015	1.50	4.000 ± 0.015	1.36
		10.000 ± 0.023	1.90	10.000 ± 0.023	1.93
	Cocoa matrix	1.000 ± 0.006	1.20	1.000 ± 0.006	2.09
		4.000 ± 0.015	1.50	4.000 ± 0.015	2.50
		10.00 ± 0.023	2.70	10.000 ± 0.023	2.98

[a] CV, coefficient of variation ($n = 5$).

from 1.0 to 10.0 μg/ml, respectively. Each calibration set included seven data points and each point was run at least three times. Table 5 shows the linear regression analysis of the calibration curve data. It can be concluded that the calibration curves were linear over the concentration range studied, indicating no significant deviation from linearity (r values > 0.9901). In all cases, the slopes of the calibration graphs for cocoa and aqueous standards solutions were not statistically different ($P < 0.05$). Since no matrix interferences were detected, the standard calibration technique for aqueous standards could be used for the determination of theobromine, theophylline and caffeine in cocoa samples investigated in this work.

The lowest concentration that can be quantified (LOQ) with acceptable accuracy and precision were 0.50, 0.25 and 0.50 μg/ml for theobromine, theophylline and caffeine, respectively. Furthermore, the limit of detection (LOD), defined as a signal-to-noise ratio (S/N) > 3 were 0.10 μg of theobromine/ml, 0.08 μg of theophylline/ml and 0.10 μg of caffeine/ml when 20 μl was used as injection sample volume. These values of LOQ were sufficiently sensitive to evaluate the studied analytes in the Venezuelan cocoa samples. Nevertheless, it was possible to enhance the sensitivity further by injecting larger volumes, up to 200 μl.

Finally, the proposed method were also validated by analyzing theobromine and caffeine of the same cocoa samples by this procedure and with a reference method (Pura Naik, 2001). The regression between values found by the developed procedure (y) and the reference values (x) provides for all analytes the regression equations with the regression coefficients shown in Table 6. As it can be seen, there was a good correlation between concentrations determined by the two methods.

3.3. Application

Using the proposed method, the "Criollos", "Trinitarios" and "Forasteros" cocoa samples grown in different seasons of the year and fermented during 3 and 7 days, respectively, were analyzed. The results are reported in Fig. 5. As it can be seen, the theobromine is the xanthine

Table 5
Linear correlations between peak areas and concentrations of theobromine, theophylline and caffeine, respectively

Compound	Matrix	Equation[a]	r[b]	CV of slope (%)[c]
Theobromine	Aqueous standard	$A = 43394C$	0.9967	3.12
	Cocoa matrix	$A = 1.0 \times 10^6 + 44281C$	0.9904	3.73
Theophylline	Aqueous standard	$A = 62068C$	0.9992	2.90
	Cocoa matrix	$A = 9611 + 61433C$	0.9999	2.98
Caffeine	Aqueous standard	$A = 73226C$	0.9928	3.06
	Cocoa matrix	$A = 96188 + 74078C$	0.9901	3.57

[a] A, peak area; C, concentration of each compound.
[b] r, correlation coefficient.
[c] CV, coefficients of variation of the slope ($n = 3$).

Table 6
Validation of the proposed procedure with a reference method

Compound	Equation[a]	r^2[b]	CV of slope (%)[c]
Theobromine	$y = 1.0503x$	0.9733	3.52
Caffeine	$y = 1.0155x$	0.9734	2.80

[a] y, values of methylxanthine by developed procedure; x, values of methylxanthine by reference procedure.
[b] r, correlation coefficient.
[c] CV, coefficients of variation of the slope ($n = 3$).

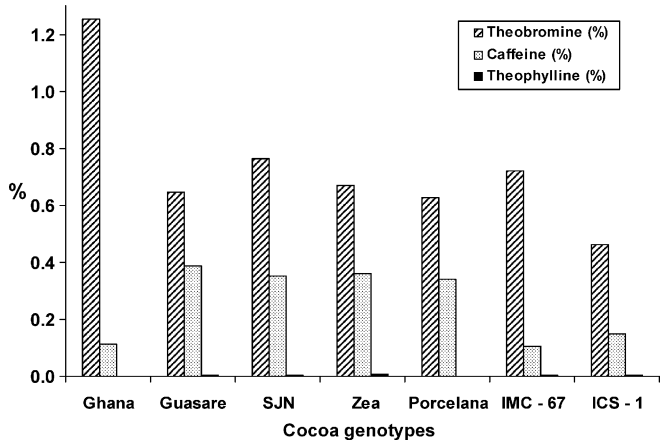

Fig. 5. Concentration of theobromine, theophylline and caffeine in different varieties of cocoa.

present in higher proportion in the cocoa beans followed by caffeine, and only by traces of theophylline. Our results agree with those reported by Pura Naik, 2001.

On the other hand, Venezuelan cocoa samples from the same harvest were analyzed on different fermentation days. The results obtained for theobromine, theophylline and caffeine are presented as a bar graph representation in Fig. 6(a)–(c), respectively. These figures show that the content for each methylxanthine is, in general, higher during the first days of fermentation, gradually decreasing while the fermentation time increases. It is important to highlight that a slight increase during the first day of fermentation for "Criollos" cocoa samples (Zea, SJN, Guasare), and during the second day for the "Forastero" (IMC-67) was observed. The high permeability of fine flavour cocoa bean testa during the first two days of fermentation might not have significant influence on the reduction of theobromine and caffeine levels in the fermented beans, because during this period the structure of cocoa bean is not yet disrupted and no alkaloids can penetrate through the testa yet (Lambert & Aitken, 2000). However, that behavior can be explained given that during the first days of fermentation, the cocoa seed pulp has not yet been extracted. It is important to remember that the methylxanthines are distributed in different parts of the cocoa seed, bean, pulp, and shell (Bucheli, Rousseau, Alvarez, Laloi, & McCarthy, 2001). Similar results report Lambert and Aitken, 2000 when evaluating the possible influence of cocoa bean testa characteristics on the flavour quality of fermented cocoa beans,

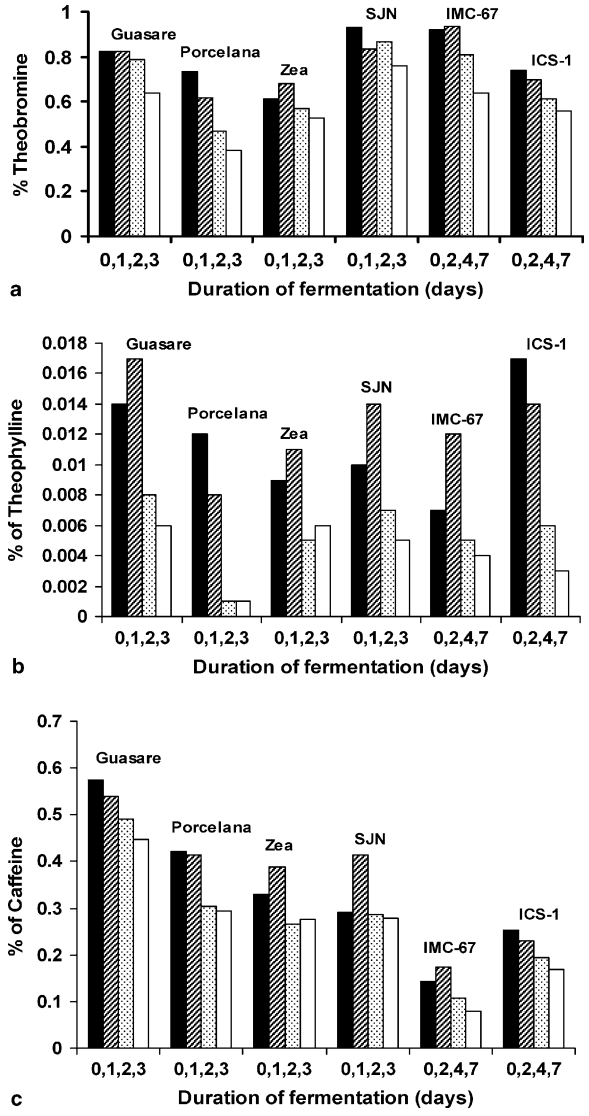

Fig. 6. Changes in methylxanthines concentration during fermentation: (a) theobromine; (b) theophylline; (c) caffeine.

compared the permeability for theobromine and caffeine of a "Forastero" and fine flavour cocoa. Results showed that bean testa of unfermented fine flavour cocoa beans was very permeable for theobromine and caffeine (95.8 and 94.8, respectively). "Forastero" showed lower permeability (24.7% for theobromine and 28.3 for caffeine). Both genotypes showed a very steep decrease of permeability after two days of fermentation and further decrease until the end of fermentation. This influence of penetration of theobromine and caffeine content, are correlated with the bitter note. Besides, it is well known that one of the purposes of fermentation of the beans is to eliminate the mucilague, to avoid the germination and to allow the biochemical reactions of the precursors of aroma of thermal origin. This fermentation procedure is accomplished in three days for "Criollos" and seven for "Trinitario" and "Forasteros" cocoa beans. Once the fermentation is completed, the reminder of the pulp has to be eliminated.

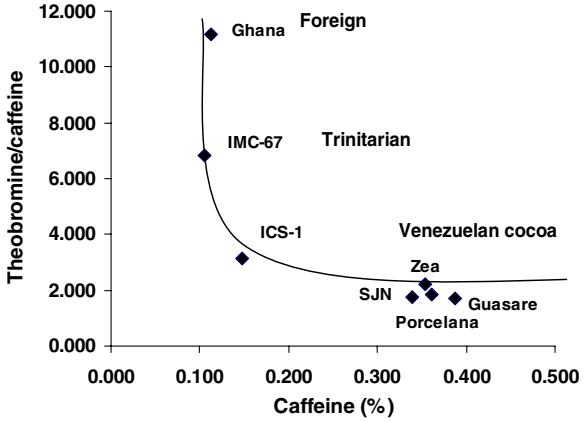

Fig. 7. Relationship between the methylxanthines content with the cocoa's genotype.

That is the reason why during the first days of fermentation there might be a methylxanthines migration from the pulp to the bean, and an increase on the concentration can be observed during the first day of the studied fermentation time.

On the other hand, it is also important to highlight that studies carried out by Davrieux, Assemat, Boulanger, and Cros (2004) showed the existence of a relationship between the methylxanthines content with the cocoa's genotype. These studies were focused in the relation theobromine/caffeine, allowing its classification in cocoa ''Forastero'', ''Trinitario'' and ''Criollo''. In this work, the correlation between the analyzed cocoa and the theobromine/caffeine relationship has been studied. Then, depending on the caffeine concentration, for each type of cocoa the relation theobromine/caffeine is shown on a graph. The obtained results are presented in Fig. 7. There we can see that the cocoa ''Criollos'' has the lower theobromine concentration and the most caffeine content, different from the ''Forastero'', which have more theobromine content and less caffeine concentration. However, the ''Trinitario'' is a hybrid and is distributed at an intermediate level.

Finally, it is important to point out that the concentration of theophylline in all cases is very low with respect to the one of theobromine and/or caffeine. However, the theophylline level it is not a parameter that allows differentiating between different types of cacao. On the other hand, the theophylline concentration is not a significant parameter in this study, because during the fermentation process the bitter flavor of the cacao fundamentally is determined by the concentration of theobromine and caffeine

4. Conclusions

In this work a fast, accurate and sensitive method was developed, allowing for the determination of the methylxanthines in cocoa bean samples. It was confirmed that there is no matrix effect, so the extracts can be assessed with a calibration curve set from the analytes aqueous standards. The use of coupled columns systems allowed to reduce the analysis time, getting to carry out the sample cleanup, the analytes separation and identification in 10 min. However, the method permits to simultaneously carry out the sample processing and the analysis step, since the analysis frequency is 8 samples/h (one injection every 7.0 min). It is important to emphasize that the pre-column used proved to have a long average lifespan. During the development of the method, approximately 1000 injections of sample cocoa extracts were made, showing no sings of deterioration. Finally, the sensitivity and accuracy reached in the propose method, so as the high analysis frequency, make this method reliable to be used as a routine method for methylxanthines analysis of a large number of cocoa samples.

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